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Final Summary Report

Award Number DAMD17-02-1-0306, Predoctoral fellowship
PI: Lea Starita
Mentor: Jeffrey D. Parvin, MD, PhD

Introduction

I have made considerable progress on the research project that I outlined in the pre-doctoral DOD breast cancer research fellowship proposal. The proposal aimed to determine targets for the ubiquitin ligase activity of BRCA1. BRCA1 (**Br**east **Ca**ncer susceptibility gene **1**) is an important tumor suppressor that protects mammary cells from malignant transformation. Recently BRCA1 has been found to have an enzymatic function as an ubiquitin ligase. Ubiquitin ligases tag other proteins with a small peptide, ubiquitin, and these tagged proteins then signal for downstream events such as protein degradation or DNA repair. Determination of the targets for ubiquitination by BRCA1 will lead to a better understanding of how this protein performs its tumor suppressor function.

The specific aims for this project are as follows:

1. Identify targets for ubiquitination by BRCA1
    A. Biochemical experiments: use of purified BRCA1-containing complexes and ubiquitin activating and conjugating enzymes for in vitro reactions to determine which proteins are ubiquitinated by BRCA1.
    B. Biological experiments: use of HCC1937 cells, which contain a truncated form of BRCA1 to test the function of the carboxy-terminus in the regulation of ubiquitination.
2. Reconstitution of BRCA1 ubiquitination in a pathway in vitro.

Key research accomplishments:

Aim 1A We developed a powerful in vitro ubiquitination system using purified ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme UbcH5c (E2), purified ubiquitin and BRCA1 co-purified with its heterodimeric binding partner BARD1 (**B**RCA1 **A**ssociated **R**ING **D**omain protein **1**). We utilized this in vitro ubiquitination system to determine substrates of BRCA1/BARD1. A candidate approach led to the discovery of γ-tubulin, HMMR and RNA polymerase II as BRCA1/BARD1 ubiquitination substrates.

First, we applied this in vitro system to an observation that we had made in which inhibition of BRCA1 in breast cells results in an amplification of centrosome number. We have determined that γ-tubulin associated with purified centrosomes is a target for BRCA1/BARD1 ubiquitination. In order to assay the importance of the ubiquitination of γ-tubulin on centrosome integrity, we used mass spectrometry to determine which lysines of the γ-tubulin molecule was ubiquitinated by BRCA1/BARD1. We then mutated the lysine 48 and/or 344 to arginine and expressed these mutant γ-tubulin genes in cells. The results show that when γ-tubulin K48R is expressed in cells centrosomes become amplified

after 48 hours. These results suggest that ubiquitination of γ-tubulin at lysine 48 by BRCA1/BARD1 is necessary for maintenance of centrosome number and integrity. Proper centrosome duplication and spindle formation are crucial to prevent chromosomal instability, a hallmark of cancer cells, and BRCA1 has a role in this process. The data supporting these statements can be found in Starita et al. 2004.

Another centrosome-associated substrate for the ubiquitin ligase activity of BRCA1/BARD1, hyaluronan-mediated motility receptor (HMMR). HMMR is a homolog of characterized centrosome and microtubule-associated proteins, tac-1 in the worm and D-TACC in the fruit fly. In humans there are four TACC (transformation-associated coiled coil) genes, TACC1, TACC2, TACC3 and HMMR. HMMR localizes to centrosomes and microtubules and interacts with dynein. Dysregulation of HMMR disrupts the mitotic spindle and progression through mitosis. HMMR is linked to BRCA1 in two ways; it was determined to be functionally associated with BRCA1 by Pearson correlation coefficient (PCC) analysis, and the *C. elegans* BARD1 (brd-1) interacts with tac-1 by yeast two hybrid analysis. BRCA1 and HMMR physically interact and the interaction is strongest in the G2 and M phases of the cell cycle. Transfection of siRNA directed against HMMR causes centrosome amplification and hypertrophy in all cell lines tested. Interestingly HMMR and BRCA1 interact genetically, but only in mammary cells. When siRNA directed against both BRCA1 and HMMR are expressed in mammary cells centrosome numbers did not change. HMMR is a substrate of BRCA1/BARD1 E3 ligase activity *in vitro* and ubiquitinated HHMR exists in cells (Figure 1). This work was performed in collaboration with the lab a Marc Vidal at the Dana Farber Cancer Institute. A manuscript describing this work has been submitted.

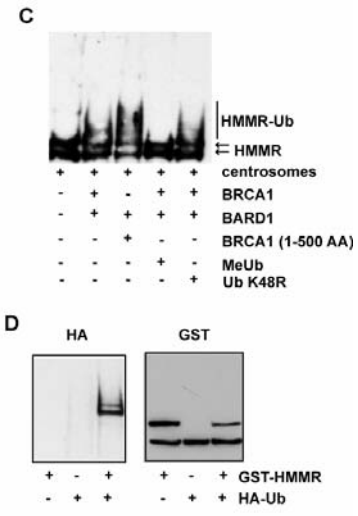

Figure 1 (C) BRCA1/BARD1-mediated polyubiquitination of HMMR *in vitro*. HMMR polyubiquitination was observed when BRCA1/BARD1 or BRCA1(1-500 AA)/BARD1 heterodimers were added to the reaction, but not when a RING-domain truncated BRCA1 isoform was used (Δ1-300 AA). (D) Detection of ubiquitinated HMMR in 293 cells. Cells were transfected with GST-HMMR and/or HA-tagged ubiquitin constructs. Lysates were glutathione-affinity purified, and analyzed by western blot for HA (antibody HA.11). The migration of the HA-tagged ubiquitin is consistent with polyubiquitinated GST-HMMR.

We found that hyperphosphorylated RNAPII is also ubiquitinated by BRCA1/BARD1 and that this ubiquitination is increased after DNA damage. Ubiquitination of RNAPII *in vitro* required that heptapeptide repeats (YS^2PTS^5PS) in the CTD of RNAPII be phosphorylated on ser5 and the reaction was dependent on the amino terminus of BRCA1 and BARD1. BRCA1 also stimulated the ubiquitination of RNAPIIO in cells and this stimulation required both amino and carboxy-termini of BRCA1 and was markedly increased after DNA damage by UV irradiation. A cancer pre-disposing mutation in BRCA1, M1775R, abolished the stimulation of RNAPII ubiquitination by BRCA1. The data for this is described in Starita et al. 2005. Another group found a decrease in RNAPIIO ubiquitination in response to DNA damage was detected when cells were transfected with siRNA against both BRCA1 and BARD1. They also noticed that 3' end processing of mRNA is inhibited by both DNA damage and BRCA1/BARD1 (Kleiman et al., 2005).

Progress for aim 1B. We are using HS578T breast cell lines that are transfected with siRNA targeted against BRCA1 to confirm whether the targets for ubiquitination found in vitro are important in vivo. Assays for immunoprecipitating ubiquitinated proteins were never sufficiently robust and are still being optimized in the lab.

Progress for aim 2. Reconstitution of BRCA1 ubiquitination function in a pathway in vitro.
We have made some progress on this aim. We had proposed in the original application that BRCA1 was a component in a DNA damage sensing mechanism whereby DNA damage would be detected by transcription by RNA polymerase II as it synthesized mRNA. BRCA1 and BARD1, present in the complex would then ubiquitinate the polymerase and signal for the initiation of damage repair. We have data, which support this model (Starita et al. 2005). Andrew Horwitz, another graduate student in the lab that is funded by a DOD/BCRP fellowship, is following up on these findings.

Taken together all of these results allowed me to receive my doctoral degree in November, 2005.

Reportable Outcomes:
Publications resulting from DAMD17-02-1-0306
Starita LM, Parvin JD. Substrates of the BRCA1-Dependent Ubiquitin Ligase. Cancer Biol Ther. 2006 Feb 4;5(2)

Starita LM, Substrates for the BRCA1/BARD1 ubiquitin ligase. Docotral Dissertation, September 9, 2005.

Pujana M.A.,* Han J.J.,* **Starita L.M.,*** Tewari M, Ahn J.S., Assmann V., ElShamy W.M., Rual J., Gelman R, Gunsalus K., Greenberg R.,[1] Bohian B., Bertin N., Ayivi-Guedehoussou N., Nathanson K.L, Weber B.L., Hill D.E., Livingston D.M., Parvin J.D., and Vidal M. (*Submitted*) A model of the BRCA1/BRCA2 network.
*co-first authors

Starita L.M.*, Horwitz A.A.*, Keogh M.C., Ishioka C., Parvin J.D., and Chiba N. (2005). BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. **J Biol Chem** 280(26):24498-505.
*co-first authors

Sankaran, S., **Starita L.M.,** Groen A.C., Ko M.J., and Parvin J.D. (2005). Centrosomal microtubule nucleation activity is inhibited by BRCA1-dependent ubiquitination. **Mol Cell Biol** (19):8656-68.

Starita LM, Machida Y, Sankaran S, Elias JE, Griffen, K, Schlegel BP, Gygi SP, Parvin JD. BRCA1-dependent ubiquitination of γ-tubulin regulates centrosome number. **Molecular and Cellular Biology.** 2004; Oct;24(19): 8457-66.

Starita LM, Parvin JD. The multiple nuclear functions of BRCA1: transcription, ubiquitination, and DNA repair. **Current Opinion In Cell Biology** 2003; *15,* 345-350.

Presentations resulting from DAMD17-02-1-0306
Starita L.M., Sankaran, S., and Parvin J.D. (2005) BRCA1-dependant ubiquitination of γ-tubulin regulates centrosome number and function. Era of Hope, Department of Defense, Breast Cancer Research Program Meeting

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Starita L.M., Sankaran, S., and Parvin J.D. (2005) BRCA1-dependant ubiquitination of γ-tubulin regulates centrosome number and function. Keystone Symposia on Ubiquitin and Signaling.

Starita L.M., and Parvin J.D. (2004) BRCA1-dependent ubiquitination of gamma tubulin regulates centrosome number. Dana Farber/Harvard Cancer Center Breast Cancer Symposium.

Starita L.M., and Parvin J.D. (2003) BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. Keystone Symposia on Molecular Targets for Cancer Therapy

Employment resulting from DAMD17-02-1-0306
I am currently a senior post-doctoral fellow in the lab of Stanley Fields at the University of Washington.

Conclusions

The most significant insight into the biology of genetic breast cancer is the recognition of BRCA1/BARD1 as an enzyme. An enzyme has a defined function and the function of BRCA1/BARD1 is to covalently modify substrates with ubiquitin. In the studies funded by this BCRP pre-doctoral fellowship we described three substrates for BRCA1/BARD1 ubiquitin ligase enzymatic activity: RNA polymerase II, γ-tubulin and HMMR. These substrates not only give insight into the etiology of breast cancer, but the more basic cellular mechanisms of transcription-coupled DNA damage repair and centrosome duplication.

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BRCA1/BARD1 Ubiquitinate Phosphorylated RNA Polymerase II*

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The breast- and ovarian-specific tumor suppressor BRCA1, when associated with BARD1, is an ubiquitin ligase. We have shown here that this heterodimer ubiquitinates a hyperphosphorylated form of Rpb1, the largest subunit of RNA polymerase II. Two major phosphorylation sites have been identified in the Rpb1 carboxyl terminal domain, serine 2 (Ser-2) or serine 5 (Ser-5) of the YSPTSPS heptapeptide repeat. Only the Ser-5 hyperphosphorylated form is ubiquitinated by BRCA1/BARD1. Overexpression of BRCA1 in cells stimulated the DNA damage-induced ubiquitination of Rpb1. Similar to the *in vitro* reaction, the stimulation of Rpb1 ubiquitination by BRCA1 in cells occurred only on those molecules hyperphosphorylated on Ser-5 of the heptapeptide repeat. *In vitro*, the carboxyl terminus of BRCA1 (amino acids 501–1863) was dispensable for the ubiquitination of hyperphosphorylated Rpb1. In cells, however, efficient Rpb1 ubiquitination required the carboxyl terminus of BRCA1, suggesting that interactions mediated by this region were essential in the complex milieu of the nucleus. These results link the BRCA1-dependent ubiquitination of the polymerase with DNA damage.

BRCA1, the breast- and ovarian-specific tumor suppressor protein, has been found to regulate a number of processes central to the normal function of the cell, including transcription, chromatin dynamics, homologous recombination, and other forms of DNA damage repair (1, 2). Because BRCA1 has been found associated with a wide range of proteins involved in these processes, it may function as a scaffold, organizing effector proteins in a context-dependent manner. However, when BRCA1 is associated with the BARD1 protein, it is also an enzyme, an E3 ubiquitin ligase (3, 4). The realization that BRCA1 is an enzyme establishes the necessity of identifying its substrates in order to understand how the ubiquitination activity impacts these processes in the cell.

BRCA1 and BARD1 are associated with the messenger RNA-synthesizing polymerase in a complex known as the RNA polymerase II holoenzyme (holo-pol)[1] (5–7). One function for BRCA1 in this holo-pol complex appears to be as a coactivator of transcription, because it has been shown that BRCA1 stimulates the activation signal of p53, NF-κB, and others (8–13). Previously, we modeled that the BRCA1 and BARD1 in the holo-pol complex may ubiquitinate the transcribing RNA polymerase II (RNAPII) when it encounters DNA damage, and we also suggested that this ubiquitination event would stimulate the repair process (14, 15).

Rpb1 is the largest subunit of RNAPII, and its carboxyl-terminal domain (CTD) is highly conserved, consisting of multiple repeats (27 in budding yeast, 52 in humans) of the heptapeptide YSPTSPS. Serines 2 (Ser-2) and 5 (Ser-5) of multiple repeats are phosphorylated co-transcriptionally, Ser5*p predominating at the promoter and Ser2*p in the coding sequence (16, 17). In response to DNA damage Rpb1 is also ubiquitinated, an event associated with changes in concentration of both the hypophosphorylated and the hyperphosphorylated Rpb1 (18). In budding yeast, the Rsp5 E3 ligase ubiquitinates Rpb1 independent of its phosphorylation state (19, 20). In higher eukaryotes the ubiquitin ligase(s) that mediate this modification of RNAPII are unknown, and it is possible that multiple factors mediate the reaction. Because BRCA1 and BARD1 are associated with RNAPII in the holo-pol complex (6), BRCA1 is a reasonable candidate for the RNAPII ubiquitin ligase. In addition, after DNA damage BRCA1 and BARD1 also associate with the polyadenylation cleavage factor CstF (21), known to interact with RNAPII via Rpb1 hyperphosphorylated on Ser-2 (Ser2*p) of the YSPTSPS heptapeptide repeats (22, 23). These results led us to speculate that a substrate for BRCA1-dependent ubiquitination could be the Ser2*p form of Rpb1.

In these experiments we tested whether BRCA1 in association with BARD1 could ubiquitinate RNAPII. We found that hyperphosphorylated RNAPII serves as a substrate for the BRCA1-dependent ubiquitination activity, and we found that overexpression of BRCA1 in cells stimulates the DNA damage-induced ubiquitination of hyperphosphorylated RNAPII. Strikingly, the ubiquitination reaction, when tested both *in vitro* and *in vivo*, was enhanced not by Ser2*p of the heptapeptide

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[1] The abbreviations used are: holo-pol, RNA polymerase II holoenzyme; BRCA1, breast cancer gene 1; BARD1, BRCA1-associated RING domain protein 1; CTD, Rpb1 carboxyl-terminal domain; GST, glutathione *S*-transferase; RNAPII, RNA polymerase II; Rpb1, RNA polymerase II subunit 1; Ser2*p, phosphorylated serine 2 of YSPTSPS; Ser5*p, phosphorylated serine 5 of YSPTSPS; HEK, human embryonic kidney; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin.

repeat but rather by Ser5*p. These results thus identify a substrate for ubiquitination by BRCA1/BARD1 that is correlated with the cellular response to DNA damage.

MATERIALS AND METHODS

Protein Purification—The expression and purification of BRCA1 and BARD1 from baculovirus-infected insect cells has been described, along with a description of the purification of the ubiquitination factors E1 and UbcH5c E2 (24). The core RNAPII was purified from calf thymus using an established protocol (25). The budding yeast Rpb1 CTD was expressed as a hexahistidine and GST fusion (26) and purified by nickel-nitrilotriacetic acid chromatography using standard techniques. Ubiquitin was obtained from a commercial vendor (Sigma).

The yeast Kin28, Ctk1, and Srb10 kinases were each expressed in *Saccharomyces cerevisiae* as HA-tagged fusion proteins. Active kinases were purified by immunoprecipitation using the 12CA5 monoclonal antibody specific for the HA tag (27, 28).

Human TFIIH was purified from HEK-293 cells as described (29). In brief, ~10^{12} cells were collected over a period of several months, and a whole cell extract was prepared for each. The whole cell extracts were bound to a Biorex70 matrix at 0.15 M KOAc in buffer A (20 mM Hepes, pH 7.9, 1 mM EDTA, 5% glycerol, 3 mM dithiothreitol), washed at 0.3 M KOAc, 0.6 M KOAc, and the peak was collected at 1.5 M KOAc. At each column step, TFIIH-containing fractions were identified by Western blotting using antibodies specific to the 89-kDa ERCC-3 subunit of TFIIH. The 1.5 M KOAc peak fraction was dialyzed to 0.1 M KCl in buffer A, bound to a DEAE fast flow matrix, and the protein peak at 0.3 M KCl was collected and dialyzed to 0.1 M KCl. The protein was bound to a 2-ml BioScale-Q column (Bio-Rad Laboratories), and protein was eluted in a gradient from 0.1 to 1.0 M KCl. TFIIH-containing fractions were subjected to gel filtration using a Superdex-200 (HR16/60; Amersham Biosciences) column in 0.3 M KCl in buffer A. The TFIIH migrated at a volume consistent with a 700-kDa complex, and samples were dialyzed in 0.1 M KCl in buffer A.

In Vitro Ubiquitination Assay—Purified RNAPII (10 ng) or 300 ng of GST·CTD/reaction were phosphorylated using purified human TFIIH or 12CA5 resin-bound HA-Ctk1, HA-Srb10, or HA-Kin28 kinase complexes using the following reaction conditions: 10 mM HEPES (pH 7.9), 0.5 mM EDTA, 5% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 5 mM NaF, 10 μCi of [γ-^{32}P]ATP. ^{32}P-labeled RNAPII was then added to ubiquitination reactions that contained 100 ng of FLAG-BRCA1/BARD1 (25 nM) or truncations of BRCA1 co-purified with BARD1 (24), 100 ng of His_6-E1 ubiquitin ligase (40 nM), 1.5 μg of His_6-UbcH5c (4 μM), and 2 μg of ubiquitin (12 μM) in the following reaction conditions: 10 mM HEPES, pH 7.9, 5% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 5 mM NaF, 2 mM ATP. All reactions were incubated at 37 °C for 30 min. The reactions were stopped by addition of sample buffer and resolved by SDS-PAGE.

Plasmid Construction—pcDNA3-HA-BRCA1(Δ775–1292)-C61G was constructed as follows. The plasmid pcDNA3-HA-BRCA1(Δ775–1292) has been described previously (30). A fragment containing the mutation C61G was amplified from an adenovirus shuttle vector that expresses full-length HA-BRCA1-C61G (31). PCR from this template used the primers 5′-ACCCCAAGCTTACCATGGCC-3′ that contains the HindIII site and 5′-TCTGTTATGTTGGCTCCTTG-3′ that is located in 3′-side of the EcoRI site of BRCA1. The PCR product was subcloned into the HindIII and EcoRI sites of pcDNA3-HA-BRCA1(Δ775–1292).

pcDNA3-HA-BRCA1(Δ775–1292) was constructed as follows. A fragment was PCR amplified from the template pcDNA3-HA-BRCA1 using the mutagenic primer 5′-GCCCTTCACCAACA*G*GCCCACAGATC-3′ and a downstream, vector-encoded primer 5′-TGACACTATAGAATAGGGCC-3′. The PCR product was used as a megaprimer with 5′-GGAAACAAAATGTTCTGCTAGCTTG-3′ to amplify a fragment encoding BRCA1 amino acids 1293–1863 containing the M1775R substitution. The second PCR product was subcloned into the NheI and EcoRV sites of pcDNA3-HA-BRCA1(Δ775–1292), thus replacing the wild-type sequence.

pcDNA3-HA-BRCA1(Δ775–1292, Δ1527–1863) was constructed as follows. The fragment containing HA-BRCA1 sequences up to residue 1526 was generated by digestion of pcDNA3-HA-BRCA1(Δ775–1292) with HindIII and SacI and then inserted into the HindIII and EcoRV sites of the vector backbone for pcDNA3-HA-BRCA1(Δ775–1292).

pCMV-Myc-ubiquitin was constructed as follows. Ubiquitin was amplified from cDNA of HeLa cells as a template using the primers 5′-GCCGAATTCGGATGCAGATCTTCGTGAAAAC-3′ and 5′-CCGCTCGAGCTAACCACCTCTCAGACGCAGG-3′ that contain 5′-EcoRI site and 3′-XhoI site. The PCR product was then subcloned into the pCMV-Myc vector (Clontech). All constructs were verified by DNA sequence.

In Vivo Ubiquitination Assay—HEK-293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and streptomycin and transfected with expression vector to express HA-BRCA1, HA-BRCA1(Δ775–1292), HA-BRCA1(Δ775–1292)-C61G, HA-BRCA1(Δ775–1292)-M1775R, and HA-BRCA1(Δ775–1292, Δ1527–1863). Two days post-transfection, cells were exposed to 20 J/m^2 of ultraviolet light and incubated with 50 μM MG132 (Sigma) in Me_2SO or Me_2SO alone for 2 h. Cell lysates were prepared in 1 ml of wash buffer (10 mM Hepes, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). For immunoprecipitation, 2.5 μl of anti-HA monoclonal antibody (HA.11; Covance), 3 μl of anti-Myc monoclonal antibody (9E10; Covance), or 7 μl of monoclonal antibody H14 and 20 μl of protein G-Sepharose beads (Amersham Biosciences) were added to each lysate. Mixtures were incubated at 4 °C overnight with rotation, the supernatant was removed, and protein beads were washed three times using 0.4 μl of wash buffer. For Western blot analysis, samples were subjected to electrophoresis in 5 or 5.5% SDS-polyacrylamide gels and immunoblotted using the monoclonal antibodies H14 or H5 (Covance), which recognize the Rpb1 CTD phosphorylated on Ser-5 or Ser-2, respectively, the anti-HA antibody HA.11, or the anti-Myc antibody 9E10.

RESULTS

BRCA1/BARD1 Ubiquitinate Hyperphosphorylated RNAPII in Vitro—BRCA1, in association with its heterodimeric partner BARD1, comprise an E3 ubiquitin ligase (3). Because BRCA1 and BARD1 associate with RNAPII (5, 7, 32), we hypothesized that RNAPII may be ubiquitinated by BRCA1/BARD1 in response to DNA damage, facilitating the repair of this damage in actively transcribed genes (14, 15).

To test this hypothesis, we utilized purified RNAPII core enzyme that had been phosphorylated *in vitro* by TFIIH as a substrate in ubiquitination reactions. Purified RNAPII exists in two forms, the IIA form, in which the Rpb1 CTD has a low level of phosphorylation, and the IIO form, in which this domain is hyperphosphorylated and has significantly shifted migration on SDS-PAGE. Phosphorylation of this RNAPII preparation by TFIIH results in the labeling of both of these forms of Rpb1 (Fig. 1*A*, *lanes 1* and *2*). This labeled RNAPII was tested in ubiquitination reactions that contained purified E1, E2 UbcH5c, E3 BRCA1/BARD1, and ubiquitin. In the complete reaction, the RNAPIIO band disappeared and a slower migrating diffuse band was observed. Under these conditions, the hypophosphorylated RNAPIIA was not modified (Fig. 1*A*, *lane 3*). These results suggest that the hyperphosphorylated RNAPII is a substrate for the BRCA1/BARD1 ubiquitin ligase.

The appearance of the slowly migrating RNAPIIO band was dependent upon the inclusion of each ubiquitination factor. Single omission of the substrate, E1, E2, E3, or ubiquitin failed to produce the slowly migrating RNAPIIO band (Fig. 1*B*). The appearance of the slowly migrating RNAPIIO band was thus consistent with modification by ubiquitination because only when all ubiquitination factors were included in reactions did this species appear (*lane 1*).

We tested whether the full 12-subunit RNAPII complex was required for ubiquitination by BRCA1/BARD1 or whether the phosphorylated CTD would suffice. The experiment of Fig. 1*B* was repeated using only the Rpb1 CTD fused to GST. This substrate was phosphorylated by purified TFIIH and [γ-^{32}P]ATP. When labeled GST·CTD was incubated with the complete reaction containing E1, E2 UbcH5c, ubiquitin and BRCA1/BARD1, the GST·CTD protein had markedly slowed migration. In this portion of the gel (>85 kDa), the resolution was imperfect, and we interpret the diffuse band with slowed migration to be consistent with the multiple additions of 8-kDa ubiquitin moieties (Fig. 1*C*, *lane 1*). The CTD of this substrate protein had no lysines to be modified by ubiquitination. We suggest that the CTD recruits the BRCA1/BARD1 E3 ligase for the ubiquitination of a separate domain of the polypeptide. These results indicate that both the 12-subunit RNAPII com-

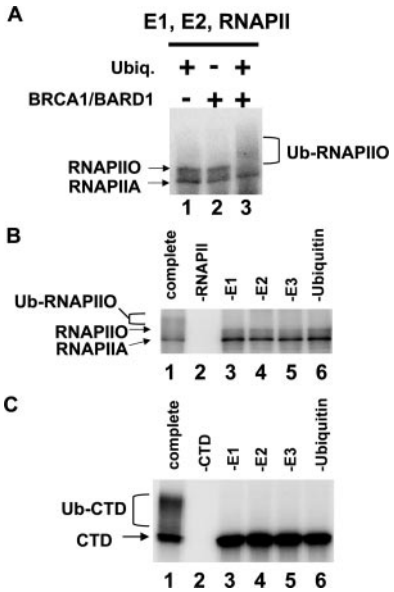

FIG. 1. **BRCA1/BARD1 ubiquitinate the Rpb1 subunit of RNAPII.** *A*, purified RNAPII was phosphorylated on the Rpb1 subunit with TFIIH and [γ-^{32}P]ATP and tested for subsequent ubiquitination using purified E1, E2 UbcH5c, E3 BRCA1/BARD1, and ubiquitin (*lane 3*). BRCA1/BARD1 and ubiquitin were included in reactions as indicated. Radiolabeled products were resolved by SDS-PAGE and identified by autoradiography. The hyperphosphorylated RNAPIIO and hypophosphorylated RNAPIIA bands are indicated. *B*, ubiquitination of RNAPII by BRCA1/BARD1 requires all of the ubiquitination factors. The complete reaction, as in *panel A*, was analyzed in *lane 1*. In *lanes 2–6* four components were included in reactions, and a different single component was omitted in each reaction. Reactions lacked RNAPII (*lane 2*), E1 (*lane 3*), E2 UbcH5c (*lane 4*), E3 BRCA1/BARD1 (*lane 5*), and ubiquitin (*lane 6*). *C*, reactions as in *panel B* were repeated except that TFIIH-phosphorylated GST·CTD was used in place of RNAPII.

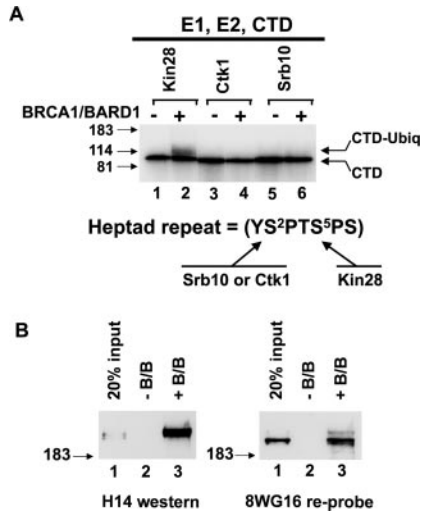

FIG. 2. **Ubiquitination of the Rpb1 CTD by BRCA1/BARD1 is stimulated by phosphorylation of the Ser-5 residue of the heptapeptide repeat.** *A*, purified GST·CTD was radiolabeled by phosphorylation with the indicated kinases. The labeling reactions create the Ser-2- or Ser-5-specific phosphopeptides, and reactions were balanced for the amount of GST·CTD and for the level of phosphorylation. After labeling, the ubiquitination reactions included E1, E2 UbcH5c, and ubiquitin. BRCA1 and BARD1 were added in reactions used in *even lanes*. *B*, purified RNAPII was subjected to affinity purification on anti-FLAG antibody containing M2-agarose beads (*lane 2*) or the same beads bound to full-length FLAG-tagged BRCA1/BARD1 protein (*B*/*B*; *lane 3*). Following binding, the matrix was washed thoroughly in buffer containing 0.3 M NaCl. Samples were analyzed by SDS-PAGE and immunoblotted with Ser5*p-specific H14 antibody (*left panel*) followed by reprobing with the RNAPIIA-specific 8WG16 antibody (*right panel*).

plex and the GST·CTD were substrates for the BRCA1/BARD1 E3 ubiquitin ligase.

The CTD used in these experiments was from the budding yeast *S. cerevisiae*, and contained 26 copies of the YSPTSPS heptapeptide. The CTD is co-transcriptionally phosphorylated *in vivo* on both Ser-2 and Ser-5. RNAPII containing unphosphorylated Rpb1 is preferentially recruited to preinitiation complexes but is phosphorylated during the transition from initiation to elongation. A Ser5*p form of the Rpb1 CTD predominates at the promoter, with Ser2*p CTD more prevalent in the coding sequence. TFIIH kinase activity is directed primarily at Ser-5 (23), with human Cdk7 and its homolog Kin28 in *S. cerevisiae* acting as the kinase in each case. The *S. cerevisiae* kinases Ctk1 and Srb10 have highest phosphorylation activity directed at Ser-2 (28). When the CTD is expressed and purified from bacteria, it is unphosphorylated, whereas RNAPII purified from eukaryotic cells is phosphorylated to different degrees on both serine positions. To test which phosphorylation event is required for ubiquitination, it was necessary to use the CTD purified from bacteria. Incubation of the CTD with each specific kinase results in differently phosphorylated products: predominantly Ser5*p when Kin28 is the kinase or Ser2*p when Ctk1 or Srb10 is used (28). We tested whether the ubiquitination activity of BRCA1/BARD1 was directed specifically at the Rpb1 CTD containing either Ser5*p or Ser2*p. In Fig. 2*A*, the GST·CTD was labeled by phosphorylation with Kin28, Ctk1, or Srb10 prior to incubation in the ubiquitination reaction. Ser5*p GST·CTD was multiply ubiquitinated in the presence of BRCA1/BARD1 (Fig. 2*A*, *lane 2*), but Ser2*p GST·CTD ubiquitination could not be detected (*lanes 4* and *6*). This result suggested that the ubiquitination of the CTD by BRCA1/BARD1 was specific for substrates containing Ser5*p.

The specificity of the BRCA1/BARD1 E3 ligase in this reaction was striking. If the heterodimer was simply binding to and ubiquitinating a long polypeptide with multiple negative charges, as in the hyperphosphorylated CTD, then we would expect little or no preference for either the Ser2*p or Ser5*p forms. Instead, the ubiquitination by BRCA1/BARD1 was specific for the Ser5*p CTD. In binding experiments using the purified BRCA1/BARD1 and purified RNAPII, we found that the BRCA1 bound to RNAPII independent of phosphorylation (Fig. 2*B*, *right panel*). This result was not surprising because it is known that BRCA1 binds to Rpb2 and Rpb12 of RNAPII (32). However, when comparing the effectiveness of the purification of RNAPII on a BRCA1/BARD1 affinity matrix, the recovery of the Ser5*p-RNAPII was more complete than was observed for the hypophosphorylated form (Fig. 2*B*, *left panel*). Thus, binding alone did not specify the ubiquitination substrate, but Ser5-specific phosphorylation enhanced both the level of binding and of ubiquitination by BRCA1/BARD1. Note that the Ser5*p form of the CTD is observed at the promoter, whereas the Ser2*p is associated with transcription elongation (17). Thus, the Ser5*p-specific modification of RNAPIIO by BRCA1/BARD1 is not consistent with targeting the elongating polymerase for ubiquitination.

BRCA1 Truncated from the Carboxyl Terminus Ubiquitinated Phosphorylated RNAPII in Vitro—The carboxyl terminus of BRCA1 (amino acids 1650–1863) associates with RNAPII via interactions with Rpb2, Rpb12, and phospho-Rpb1 subunits (7, 32). To determine whether the carboxyl terminus is required to mediate ubiquitination of RNAPII *in vitro*, we purified carboxyl-terminal truncations of BRCA1 in heterodimeric complex with full-length BARD1 (24). In addition to full-length FLAG-tagged BRCA1 (1–1863), FLAG-tagged BRCA1(1–1852), BRCA1(1–1527), BRCA1(1–1000), and BRCA1 (1–500) were coexpressed with untagged BARD1 and purified. A ΔN-BRCA1 construct (301–1863) lacking the amino-

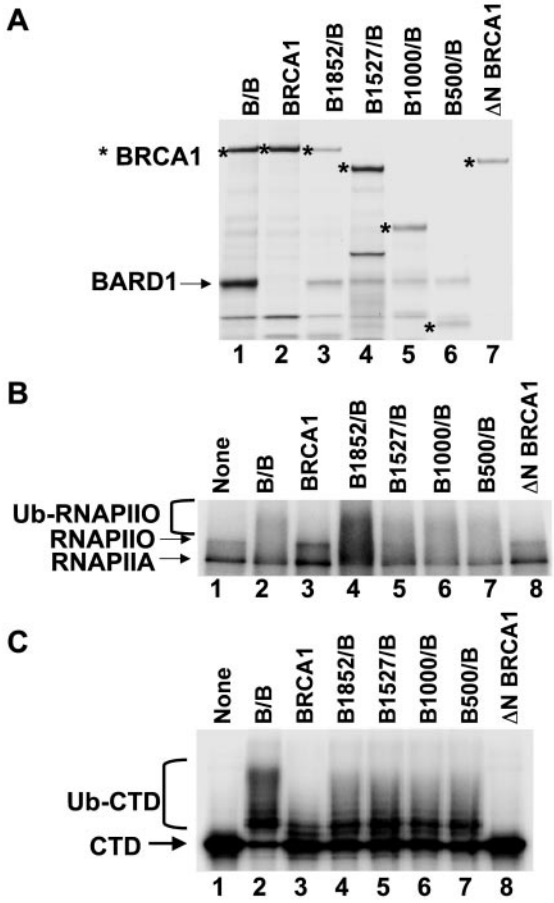

FIG. 3. **BRCA1 amino acid residues 501–1863 are dispensable for ubiquitinating phosphorylated RNAPII *in vitro*.** *A*, silver stain of a protein gel of the BRCA1/BARD1 preparations used in *panels B* and *C*. BARD1 is indicated at the side and migrated at a position consistent with a mass of 97 kDa. The BRCA1 polypeptides are marked with an *asterisk*. In each case that included BARD1, BRCA1 and BARD1 were co-expressed in insect cells and purified via an epitope tag on BRCA1. *B*, purified RNAPII, as in Fig. 1*A*, was tested as a substrate for ubiquitination using the following BRCA1 preparations: none (*lane 1*), full-length BRCA1 plus BARD1 (*lane 2*), full-length BRCA1 alone (*lane 3*), BRCA1(1–1852) plus BARD1 (*lane 4*), BRCA1(1–1527) plus BARD1 (*lane 5*), BRCA1(1–1000) plus BARD1 (*lane 6*), BRCA1 (1–500) plus BARD1 (*lane 7*), and BRCA1(301–1863) (*lane 8*). *C*, reactions as in *panel B* were repeated replacing the RNAPII complex with GST·CTD that had been labeled using TFIIH.

terminal RING domain was also purified, as was a full-length BRCA1 lacking BARD1. These constructs were balanced for BRCA1 content (Fig. 3*A*) and tested for activity in ubiquitination assays as before.

In assays using RNAPII as the substrate, ubiquitination was specific for the hyperphosphorylated Rpb1. Similar specificity was observed for all constructs tested with the exception of BRCA1 alone and the ΔN construct, which had no detectable activity. Thus, BARD1 and the BRCA1 RING domain were each required for ubiquitination of RNAPII (Fig. 3*B*). The absence of activity seen with BRCA1 lacking BARD1 is consistent with previously published results. BARD1 is required for a high level of ubiquitination activity of BRCA1, and the isolated RING domains of each protein have been shown to have low levels of ubiquitination activity *in vitro* (3, 33, 34). However, the ubiquitination activity of BRCA1 is significantly potentiated by its interaction with BARD1 (3, 4), and structural studies of the amino terminus of BRCA1 and BARD1 reveal extensive interaction between these domains (35). The ΔN construct lacks a RING domain and was thus expected to lack ubiquitination activity.

All of the active truncations of BRCA1 specifically ubiquitinated the hyperphosphorylated form of RNAPII, whereas the hypophosphorylated form was relatively unmodified (Fig. 3*B*). We had previously hypothesized that the carboxyl terminus of BRCA1 mediates the specificity of its association with RNAPII because this domain of BRCA1 activates transcription (36–38) and because it binds to two RNAPII subunits (32). Efficient ubiquitination of RNAPII, however, was observed even when the ubiquitin ligase was a BRCA1 truncation that lacked the carboxyl terminus, suggesting that the function of the BRCA1 carboxyl-terminal transcription activation domain is unrelated to its ubiquitination of phosphorylated RNAPII by BRCA1.

The RNAPII ubiquitination assay yields a qualitative result, indicating that hyperphosphorylated Rpb1 is a substrate for the ubiquitination activity of BRCA1/BARD1. We repeated the experiment using TFIIH-phosphorylated CTD (Ser5*p) as a substrate, and we found that there were no differences in the degree of ubiquitination obtained with the BRCA1 carboxyl-terminal truncations (Fig. 3*C*). Under these more sensitive conditions, weak ubiquitination was evident when BRCA1 lacking BARD1 was included in reactions (Fig. 3*C*, *lane 3*), whereas the ΔN construct had no ubiquitination activity (Fig. 3*C*, *lane 8*). Therefore, *in vitro*, the carboxyl terminus of BRCA1 is not required for ubiquitination of hyperphosphorylated RNAPII or Ser5*p-phosphorylated CTD.

BRCA1 Ubiquitinated Phosphorylated RNAPII in Vivo—We next asked whether BRCA1 could ubiquitinate hyperphosphorylated RNAPII *in vivo*. We transfected HEK-293T cells with plasmids encoding HA epitope-tagged BRCA1 and Myc epitope-tagged ubiquitin. Transfected cell lysates were immunoprecipitated using antibody specific to the Myc epitope, thus purifying ubiquitinated proteins, and then immunoblots were probed using antibodies specific to RNAPII. The immunoblot was stained with the monoclonal antibody H14, which specifically binds to RNAPII phosphorylated on Ser-5 of the heptapeptide repeat in the CTD (18). The lysate (*input*) contained a phosphorylated RNAPII large subunit that migrated at a position consistent with 240 kDa (Fig. 4*B*, *lane 1*). Background levels of ubiquitinated phospho-RNAPII were detected in cells transfected with vector alone (*lane 2*). It is established that hyperphosphorylated RNAPII becomes ubiquitinated following ultraviolet (UV) irradiation of cells (18, 39–41), and we detected the UV-dependent ubiquitination of RNAPII (Fig. 4*B*, *lane 5*). Most of the ubiquitinated species migrated on protein gels with a very small shift relative to the unmodified species (compare *lanes 5* and *1*), and this would be expected for a low number of ubiquitin moieties (about 8 kDa each) bound to a 240-kDa polypeptide. The resolution of these species was poor by SDS-PAGE, but we consistently observed stimulated recovery of the hyperphosphorylated Rpb1 band due to ubiquitination after UV irradiation. In addition, a diffuse band of ubiquitinated species was observed shifted at slower migration that we interpret to be multiply ubiquitinated RNAPIIO.

Transfection of full-length BRCA1 had minimal effect on RNAPII ubiquitination status (Fig. 4*B*, *lanes 3* and *6*). We had previously observed that overexpression of full-length BRCA1 dysregulated normal BRCA1 complex formation, presumably by altering the cell cycle (30). In those experiments, expression of a BRCA1 with an internal deletion, HA-BRCA1(Δ775–1292), allowed us to overexpress BRCA1 and observe all of the protein complexes seen with the endogenous protein (30). This internal deletion, here called HA-BRCA1(ΔM), strongly stimulated the ubiquitination of Ser5*p-hyperphosphorylated RNAPII independent of DNA damage (Fig. 4*B*, *lane 4*, *top panel*).

UV irradiation of the cells stimulated ubiquitination of phospho-RNAPII (Fig. 4*B*, *lanes 5* and *6*), and in UV-irradiated

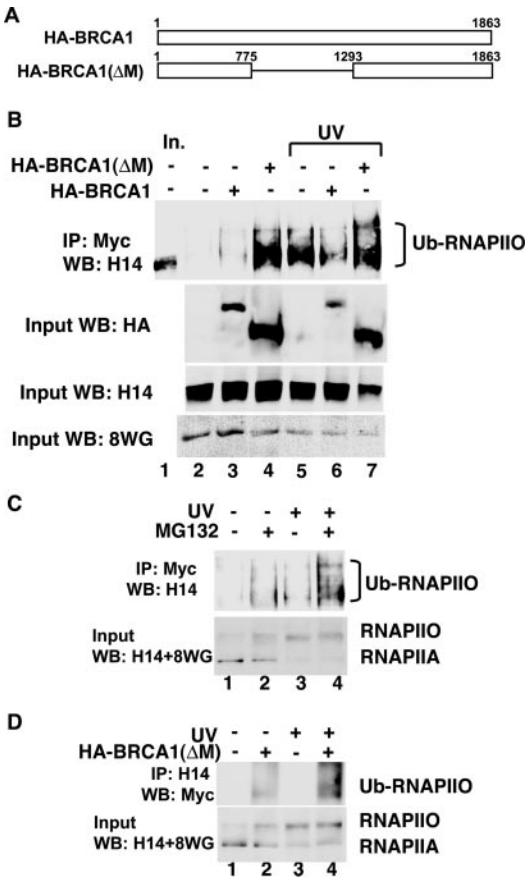

FIG. 4. **BRCA1 ubiquitinates phosphorylated RNAPII *in vivo*.** *A*, schematics are shown of the full-length BRCA1 and of the BRCA1 mutant. *B*, HEK-293T cells were transfected with vectors to express HA-BRCA1 full-length (*lanes 3*, *6*), HA-BRCA1(ΔM) (*lane 4*, *7*) and Myc ubiquitin (*lanes 1–7*). Cells were treated with 20 J/m^2 UV irradiation (*lanes 5–7*) and 50 μM MG132 (*lanes 1–7*). Lysates were immunoprecipitated by anti-Myc antibody (*lanes 2–7*). Immunoblots were stained with H14 monoclonal antibody to recognize the Ser5*p version of RNAPIIO (*top panel*). *Input* samples were immunoblotted and stained with H14, anti-HA antibody, and 8WG16 (*8WG*), the last to detect unphosphorylated RNAPII. The *input* sample (*lane 1*) was only included in the *top panel*. *C*, HEK-293T cells were transfected with vectors to express HA-BRCA1(ΔM) (*lanes 1–4*) and Myc ubiquitin (*lanes 1–4*) and treated with 20 J/m^2 UV (*lanes 3*, *4*) in the presence of 50 μM MG132 (*lanes 2*, *4*) as described under "Materials and Methods." Lysates were immunoprecipitated using an anti-Myc antibody, and immunoblots were stained for Ser5*p-RNAPIIO using antibody H14 (*top panel*). *Input* samples were stained with antibodies 8WG16 and H14 to detect hypophosphorylated RNAPIIA and Ser5*p-RNAPIIO in the samples (*bottom panel*). *D*, HEK-293T cells were transfected with vectors to express Myc ubiquitin (*lanes 1–4*) and also HA-BRCA1(ΔM) (*lanes 2* and *4*) and subjected to UV irradiation (*lanes 3* and *4*). Lysates were immunoprecipitated using the Ser5*p-specific H14 antibody and immunoblots probed with the Myc-specific antibody to detect ubiquitin (*top panel*). *Input* samples were immunoblotted using H14 and 8WG16 antibodies (*bottom panel*).

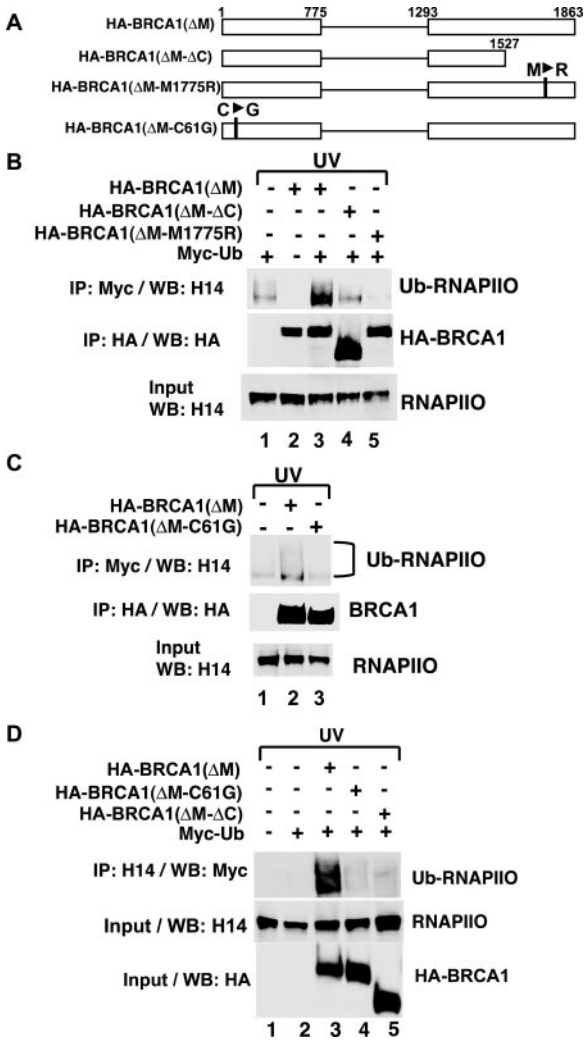

FIG. 5. **Disease-associated mutations of BRCA1 and deletion of the BRCA1 carboxyl terminus abolish BRCA1-dependent ubiquitination of phospho-RNAPII *in vivo*.** *A*, designs of BRCA1 deletion mutants and point mutations are diagrammed. *B*, BRCA1(ΔM-ΔC) and BRCA1(ΔM-M1775R) are ineffective in stimulating the ubiquitination of phosphorylated RNAPIIO. HEK-293T cells were transfected with vectors to express HA-BRCA1(ΔM) (*lanes 2*, *3*), HA-BRCA1(ΔM-ΔC) (*lane 4*), HA-BRCA1(ΔM-M1775R) (*lane 5*), and Myc ubiquitin (*lanes 1*, *3–5*). Cells were irradiated with 20 J/m^2 of UV light in the presence of 50 μM MG132, and lysates were immunoprecipitated by anti-Myc monoclonal antibody (*top panel*) or anti-HA monoclonal antibody (*middle panel*). Matching *input* samples were analyzed in the *bottom panel*. Immunoblots were stained with H14 monoclonal antibody specific for the Ser5*p-modified RNAPIIO or anti-HA monoclonal antibody as indicated. *C*, BRCA1(ΔM-C61G) was ineffective in stimulating the ubiquitination of RNAPIIO. HEK-293T cells were transfected with vectors to express HA-BRCA1(ΔM) (*lane 2*), HA-BRCA1(ΔM-C61G) (*lane 3*), and Myc ubiquitin (*lanes 1–3*). Cells were treated with 20 J/m^2 of UV irradiation in the presence of 50 μM MG132. Lysates were immunoprecipitated and immunoblotted as indicated. *D*, HEK-293T cells were transfected with plasmids expressing Myc ubiquitin (*lanes 2–5*), HA-BRCA1(ΔM) (*lane 3*), HA-BRCA1(ΔM-C61G) (*lane 4*), and HA-BRCA1(ΔM-ΔC) (*lane 5*). Cells were irradiated in the presence of MG132 as above, and lysates were immunoprecipitated using the H14 monoclonal antibody (*top panel*). *Input* lysates were analyzed in the bottom two panels, and immunoblots were probed as indicated.

HA-BRCA1(ΔM)-expressing cells a significant increase in the intensity of the slowly migrating band was observed (*lane 7*) that we interpret to be multiply ubiquitinated RNAPIIO. These results indicate that overexpression of BRCA1(ΔM) stimulated ubiquitination of Ser5*p-Rpb1 independent of, but qualitatively modified by, DNA damage. When we tested the H5 monoclonal antibody that specifically binds to Ser2*p RNAPII or the 8WG16 monoclonal antibody that specifically recognizes hypophosphorylated RNAPII on immunoblots, ubiquitinated RNAPII was not detected (data not shown). These results were consistent with the *in vitro* experiments (Fig. 2) in which Ser-5 phosphorylation of the RNAPII CTD specifically stimulated its ubiquitination by BRCA1/BARD1. These results were also consistent with the previously established ubiquitination of Ser-5-phosphorylated RNAPIIO after UV-induced DNA damage (18, 40).

The consequences of BRCA1-dependent ubiquitination are unclear. BRCA1/BARD1 have been shown to direct the linkage of ubiquitin chains via either lysine 6, lysine 48, or lysine 63 isopeptide bonds (4, 42). Appending ubiquitin chains via lysine

48 target the substrate for proteasome-mediated degradation; thus BRCA1/BARD1 ubiquitination may in some cases not lead to protein degradation. We tested whether inhibition of the proteasome, using MG132, could stabilize the ubiquitinated phospho-RNAPII. Proteasome inhibition resulted in longer chains of ubiquitin appended on the Rpb1 subunit of RNAPIIO (Fig. 4*C*, *lane 4*, *top panel*), suggesting that BRCA1-dependent ubiquitination may cause degradation of RNAPIIO. Interestingly, UV irradiation of cells resulted in a shift in the polymerase from RNAPIIA to RNAPIIO (Fig. 4*C*, *bottom panel*), a phenomenon that has been observed previously (18). Although quantitation using two different antibodies in immunoblots is imprecise, this result suggests that phosphorylation of Rpb1 to Ser5*p is a generalized response after DNA damage. Although proteasome inhibition stabilized the recovery of ubiquitinated RNAPIIO (*lanes 3* and *4*), the amount of RNAPIIO in the lysate was not markedly increased (Fig. 4*C*, *lanes 3–4*, *bottom panel*). We infer from this result that only a fraction of the total RNAPII is targeted for degradation following BRCA1-dependent ubiquitination.

Repeating the experiment, but using the H14 antibody to immunoprecipitate the RNAPIIO and the anti-Myc antibody on immunoblots to detect the ubiquitin, revealed that HA-BRCA1(ΔM) expression stimulated the appearance of ubiquitinated RNAPIIO (Fig. 4*D*, *lane 2*). As in *panel B*, expression of HA-BRCA1(ΔM) in UV-irradiated cells resulted in the recovery of higher levels of ubiquitinated RNAPIIO (Fig. 4*D*, *lane 4*). Compared with anti-Myc ubiquitin immunoprecipitation, use of the H14 antibody reproducibly yielded lower amounts of ubiquitinated RNAPIIO, even after UV irradiation. We interpret this lower level to be due to less effective immunoprecipitation reactions with the latter antibody.

We have previously shown that BRCA1 is a component of RNAPII holo-pol, and the carboxyl terminus of BRCA1 is important for this association (5, 6). In the *in vitro* assays in this study (Fig. 3), the carboxyl terminus of BRCA1 was not required for ubiquitination of the polymerase. However, in the complicated environment of a cell, the carboxyl-terminal-mutated BRCA1 might not associate with the polymerase and thus not ubiquitinate it. We examined whether the carboxyl terminus of BRCA1 affected ubiquitination of phospho-RNAPII in tissue culture cells. We found that overexpression of BRCA1 lacking its carboxyl terminus resulted in only background levels of ubiquitinated RNAPIIO (Fig. 5*B*, compare *lanes 1–4*). We thus conclude that in cells the carboxyl terminus of BRCA1 is important for the UV damage-induced ubiquitination of RNAPIIO.

We also tested whether a specific missense mutation associated with breast cancer affects the ubiquitination of RNAPIIO. The disease-associated missense mutation M1775R in the BRCT domain of the carboxyl terminus of BRCA1 ablates the double strand break repair and transcription activation function of BRCA1 (43). BRCA1 proteins containing the M1775R mutation do not bind to histone deacetylases (44), BACH1 (45), and the transcriptional co-repressor CtIP (46, 47). As shown in Fig. 5*B*, expression of BRCA1 with M1775R did not stimulate the ubiquitination of phosphorylated RNAPII (Fig. 5*B*, *lane 5*, *top panel*). Although the mutation of BRCA1 at residue M1775R decreases the stability of the protein (48), the expression level of the HA-BRCA1(ΔM-M1775R) was equal to that of HA-BRCA1(ΔM) (Fig. 5*B*, *middle panel*). Furthermore, the M1775R mutation disrupted BRCA1 binding to RNAPIIO (Fig. 6). In transfected cells, immunopurification of HA-BRCA1(ΔM) also purified Ser5*p Rpb1 (Fig. 6, *lane 2*). Deletion of the carboxyl terminus of BRCA1 or the BRCA1 protein containing a missense mutation resulted in significantly decreased binding to RNAPIIO (Fig. 6, *lanes 3* and *4*). Thus, an intact carboxyl terminus was required for BRCA1 to bind to RNAPIIO. These data suggest that ubiquitination of phosphorylated RNAPII by BRCA1 in response to DNA damage requires an intact BRCT domain.

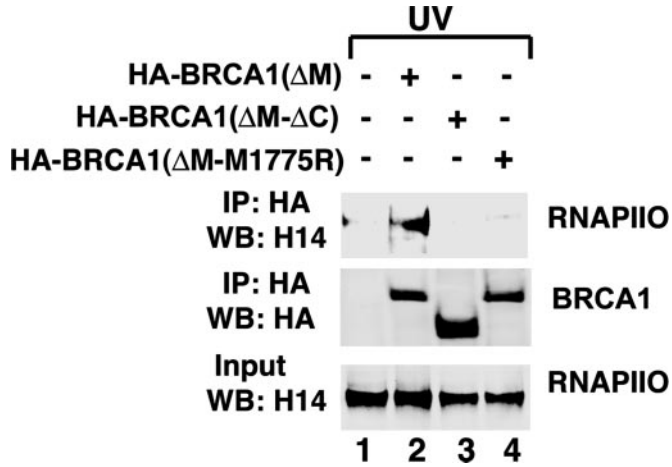

FIG. 6. **Wild-type BRCA1 binds to RNAPIIO.** HEK-293T cells were transfected with plasmids for the expression of HA-BRCA1(ΔM) (*lane 2*), HA-BRCA1(ΔM-ΔC) (*lane 3*), HA-BRCA1(ΔM-M1775R) (*lane 4*), and Myc-ubiquitin (*lanes 1–4*) and treated with 20 J/m^2 UV (*lanes 1–4*). Lysates were immunoprecipitated using anti-HA epitope antibody (*top* and *middle panels*) and probed for immunoblots as indicated. *Input* samples were analyzed in the *bottom panel*.

The active site of BRCA1 for ubiquitin ligase activity is encoded in the RING domain of the amino terminus of the protein. Missense mutation of one of the zinc-coordinating residues, C61G, results in an inactive E3 ubiquitin ligase even in the presence of wild-type BARD1 (3, 34, 35). In patients, inheritance of this missense mutation is associated with breast cancer (49, 50). Expression of HA-BRCA1(ΔM) containing the C61G missense mutation did not stimulate the ubiquitination of phosphorylated RNAPII (Fig. 5*C*, *top panel*).

The experiment in Fig. 5*C* was repeated, but the immunoprecipitating antibody was the Ser5*p-specific H14, and ubiquitinated species were detected using the Myc-specific antibody on immunoblots. As before, we observed that HA-BRCA1(ΔM) expression stimulated the recovery of ubiquitinated RNAPIIO (Fig. 5*D*, *lane 3*). Further, expression of BRCA1 variants containing the missense mutation C61G (*lane 4*) or a carboxyl-terminal truncation (*lane 5*) failed to stimulate the ubiquitination of RNAPIIO. As in Fig. 4*D*, this immunoprecipitation reaction was weaker than when the Myc antibody was used, and we only detected the ubiquitinated species when HA-BRCA1(ΔM) was expressed. Taken together, the data in Figs. 4 and 5 indicate that BRCA1 stimulates the ubiquitination of Ser5*p RNAPII after UV irradiation.

DISCUSSION

Identification of the substrates for BRCA1-dependent ubiquitination activity is important for understanding how mutation of BRCA1 is associated with loss of tumor suppression activity. The currently identified substrates include histone proteins, p53, Fanconi anemia protein D2, and centrosomal proteins including NPM1 and γ-tubulin (24, 51–54). Among these, only the modification of γ-tubulin by BRCA1/BARD1 has been shown to affect the biology of breast cells. It has been shown that failure to ubiquitinate γ-tubulin results in centrosome amplification (24). The BRCA1/BARD1 proteins are known to regulate multiple processes in the cell, including transcription, DNA repair, and centrosome dynamics (5, 55–59). Although the ubiquitination of γ-tubulin may in part ex-

plain the BRCA1-dependent regulation of centrosome dynamics, it was unclear whether the BRCA1-dependent ubiquitination activity also regulates the transcription and DNA repair function of BRCA1.

We had proposed that the BRCA1-dependent ubiquitination activity may function in DNA repair by modification of RNAPII that transcribes DNA near a lesion (14, 15). This proposed role for BRCA1 in transcription-coupled repair could be important following UV damage or double strand breaks. One prediction of this model was that BRCA1/BARD1 ubiquitination activity would be targeted to the elongating, hyperphosphorylated form of RNAPII. Actively transcribing RNAPII is phosphorylated on Ser-5 proximal to the promoter and on Ser-2 further downstream (23). Thus, the principal form of RNAPII that elongates through a gene is the Ser2*p form, which we now show is not a substrate for BRCA1/BARD1. The model that BRCA1-dependent ubiquitination directly links transcription elongation to repair is thus not supported. Instead, we found that Ser-5 phosphorylation of RNAPII is a generalized response to UV irradiation, and BRCA1-dependent ubiquitination modifies the RNAPIIO. It has been observed that transcriptionally engaged RNAPII does become phosphorylated on Ser-5 by the action of extracellular signal-regulated kinases 1 and 2 (60). The data are most consistent with a model whereby DNA damage causes phosphorylation of a subpopulation of RNAPII, followed by ubiquitination by BRCA1/BARD1 and subsequent degradation at the proteasome.

In these experiments we found that overexpression of BRCA1 in cells could stimulate the damage-induced ubiquitination of RNAPII. When we inhibited BRCA1 expression by transfection of short interfering RNA specific for BRCA1, we did not observe a decrease in ubiquitination of RNAPII.[2] We interpret these results to indicate that one or more other ubiquitin ligases can execute this function. Several other factors have been implicated in the ubiquitination of RNAPII, including Cockayne syndrome proteins CSA and CSB (60, 61). Even though other factors can also ubiquitinate RNAPII, our results overexpressing BRCA1 clearly indicate that it participates in this process.

In summary, we found in this study that BRCA1/BARD1 ubiquitinate RNAPII hyperphosphorylated via Ser-5 of the heptapeptide repeat. Rpb1 was multiply ubiquitinated. In experiments using highly purified factors *in vitro*, only the amino terminus of BRCA1, containing the catalytic RING domain, was required for ubiquitination of phospho-RNAPII. The BARD1 protein was not essential, but it was highly stimulatory. In cells, overexpression of BRCA1 could stimulate the ubiquitination of hyperphosphorylated RNAPII. In contrast to the *in vitro* reactions using purified factors, in the cell the carboxyl-terminal domain was important for the DNA damage-stimulated ubiquitination of phosphorylated RNAPII by BRCA1. These results are consistent with our observations that both the amino- and carboxyl-terminal domains of BRCA1 are required for BRCA1 association with the polymerase complex.

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